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Whole Body Hyperthermia-Induced Brain Injury in Rats: Forensic Biochemical, Pathological, and Immunohistochemical Investigations

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ABSTRACT

Episodes of hyperthermia occur frequently from exposure to infections or adverse climatic changes. Such hyperthermia has been linked to various detrimental health effects. This study aimed to investigate the pathophysiological responses to acute versus subacute whole-body hyperthermia (WBH) in rats, particularly on the brain, with special reference to their medicolegal importance. Rats were randomly assigned to three equal groups. The normothermic group was kept at room temperature, the acute whole-body hyperthermic (AWBH) group was subjected to long-period WBH as a single bout of 4 hours at 43 °C, and the subacute whole-body hyperthermic (SWBH) group was subjected to repeated short-period WBH;2 hours daily for 7 successive days at 43 °C. A significant increase in body temperature and a significant decrease in body weight were recorded in both the acute and subacute hyperthermic groups. However, significant elevations in the serum glucose and cortisol levels and in the brain malondialdehyde and 8-hydroxy-2'-desoxyguanosine levels, with a significant reduction in brain total antioxidant capacity, were observed only in the AWBH group. Moreover, exposure to WBH induced various degrees of pathological changes, along with positive immune reactivity for heat shock protein 70 and glial fibrillary acidic protein in the cerebrum and cerebellum of rats in both hyperthermic groups. Overall, WBH induced various adverse health effects, mediated by the induction of oxidative damage, particularly following acute exposure. Hence, these findings provide evidence that acute exposure to long-term WBH may markedly exacerbate brain injury more than repeated short-term exposure, reflecting the role of the adaptive mechanisms to repeated heat exposure. Interestingly, the obtained findings may be valuable in the forensic antemortem and postmortem diagnosis of WBH and heat-related deaths.

Keywords: Hyperthermia, Brain, Cortisol, Malondialdehyde, Total antioxidant capacity, HSP70 and GFAP.

INTRODUCTION

Across the globe, the incredible climatic changes result in an increment in the frequency, duration, and intensity of heat waves, which are among the most serious natural hazards that mediate numerous heat stress conditions and increase the prevalence of heat-related illness (Kovats and Hajat, 2008; Allan *et al.,* 2020).

Heat stress (HS) causes a variety of physiological and pathological responses, including heat adaptation, hyperthermia, metabolic disorders, weight loss, and hormonal imbalance (Lee *et al.,* 2015; Brugaletta *et al.,* 2022). Hyperthermia is a thermal stress syndrome that is defined as an elevation in the core body temperature exceeding 40 °C and may be accompanied by sweating, fatigue, headache, flushing, nausea, tachycardia, muscle cramps, agitation, oliguria, delirium, seizures, and coma (Cheshire, 2016). Importantly, hyperthermia may occur due to numerous infective or non-infective causes, however high environmental temperatures and global warming are considered the major causes of hyperthermia and heat-related illness in both humans and animals (Mustafa *et al.,* 2008; Walter and Carraretto, 2016).

Hyperthermia triggers thermoregulatory responses such as sweating, enhances cardiac output, and redistribution of blood flow to lower the body temperature (Argaud *et al.,* 2007). Meanwhile, a quick but temporary synthesis of heat shock proteins (HSPs) is also a defensive mechanism to minimize cellular damage caused by HS (Biedenkapp and Leon, 2013). However, if physiological adaptation to HS is disturbed, the core body temperature rises rapidly, resulting in impairment of thermoregulation and physiological functions of the body, as well as systemic inflammatory responses and multiple-organ dysfunction (Epstein and Yanovich, 2019; Nakamura *et al.,* 2022).

Frequent exposure to heat episodes causes numerous detrimental health issues (Ebi *et al.,* 2021). Hyperthermia exerts direct cytotoxic effects by interfering with cell cycle, protein and DNA synthesis, electrolyte homeostasis, membrane integrity and membrane transport proteins, and may cause cell death, either directly or by initiating apoptotic pathways (Roti Roti, 2008; Hou *et al.,* 2014).

The brain is extremely sensitive and vulnerable to any changes in temperature. Mounting evidence suggests that HS predominantly harms the central nervous system (CNS), even with a slight increase in temperature and a short duration of exposure (Lee *et al.,* 2015; Walter and Carraretto, 2016; Chauhan *et al.,* 2017; 2021).

Numerous studies reported that exposure to high temperature affects brain function and structure, causing delirium, spasms, brain atrophy, neuronal loss, neurogenic deficits and cognitive impairment in experimental animals and humans (Lee *et al.,* 2015; Cedeño Laurent *et al.,* 2018; Chauhan *et al.,* 2021). Various pathomorphological alterations were observed in the brain following exposure to HS. Importantly, brain oedema is the most noticeable clinical feature following hyperthermia, along with congestions, haemorrhages, ischemia, thrombosis, degenerative changes, necrosis,

inflammation, and gliosis (Rebez *et al.,* 2023).

Earlier studies explained the underlying mechanisms by which HS may induce brain damage, involving the generation of free radicals, disturbance of the blood-brain barrier, impairment of gene and protein synthesis, glial activation, neuroinflammation, and apoptosis (Chauhan *et al.,* 2021; Yan *et al.,* 2023).

Given the fact of worldwide global warming and the adverse consequences of heat stress on both animal and human health, which depend mainly on the intensity and duration of the thermal stress, the current investigation was conducted to study the impacts of single or repeated exposure to whole-body hyperthermia on physiological and metabolic functions, as well as brain structure and function. Importantly, the current investigation may provide insight for a better understanding of thermotolerance and how animals and humans may cope with hyperthermia, and it may also provide reliable forensic antemortem and postmortem evidence for hyperthermia and heat-related deaths.

MATERIALS AND METHODS
1. Animals:

The animals' care and handling were organized following the ethical guidelines, and the experimental design was approved by the International Animal Care and Use Committee, Faculty of Veterinary Medicine, University of Sadat City (Approval No. VUSC-019-1-21). Twenty-one mature, healthy male Sprague–Dawley rats (150–170 g) were acquired from the Alzyade Experimental Animals Production Center, Giza, Egypt. Rats were kept in polypropylene cages in a naturally ventilated room with standard laboratory hygienic conditions (25±2 °C, 40-45 % relative humidity, and a natural daily dark/light cycle). Rats were provided with a standard commercial diet and clean tap water throughout the entire experiment.

2. Animal grouping and Experimental design:

Rats were randomly allocated into three equal groups (n= 7).

Normothermic group (NT): Rats were maintained at room temperature (25±2 °C) and served as control group.

Acute whole-body hyperthermic (AWBH) group: Rats were subjected to long-term WBH as a single bout of 4 hours at 43 °C in an incubator.

Subacute whole-body hyperthermic (SWBH) group: Rats were subjected to short-term WBH as repeated daily bouts of 2 hours for 7 successive days at 43 °C in an incubator.

In order to exclude the impact of the diurnal cycle, rats were kept in an incubator with a blower and a ventilator, and exposure to heat started at approximately the same time (9 a.m. to 11 a.m.) every day, and rats were then returned to room temperature. During this period, animals were not provided with food or water to avoid metabolic and/or humidity increases, which may affect heat loss.

3. Samples collection and preparation:

Immediately after the termination of each respective protocol of exposure of the different groups, body temperature of both control and hyperthermic rats were measured rectally; and animals were weighed before being anaesthetized by diethyl ether.

Blood samples were collected from the retro-orbital plexus; centrifuged at 3000 rpm for 15 min for isolation of sera samples,

which were kept at -20 °C for estimation of serum glucose and cortisol levels.

Animals were euthanized by cervical dislocation and brain of each rat was instantly excised and divided into two parts: the first part was washed in an isotonic solution and homogenized in cold PBS (pH 7.4). The obtained homogenates were cold-centrifuged at 4 °C at 3000 rpm for 15 min and stored at –20 °C for further estimation of MDA, TAC, and 8-OHdG levels. The other part was preserved in neutral-buffered formalin 10% for histopathological and immunohistochemical investigations.

4. Levels of serum glucose and cortisol:

Serum glucose level was calorimetrically estimated using a diagnostic kit (CAT. No. GL1320) of Biodiagnostic Company, (Dokki, Giza, Egypt) according to the method of Trinder (1969). Serum cortisol level was measured using a commercial ELISA kit (CAT. No. MBS727040) of MyBioSource Company, (San Diego, USA).

5. Level of 8-hydroxy-2'-deoxyguanosine in brain:

The level of brain 8-OHdG was measured using a commercial rat ELISA kit (CAT. NO. MBS267513) of MyBioSource Company, (San Diego, USA), and following the procedure of de Souza-Pinto et al. (2001).

6. Levels of Malondialdehyde and total antioxidant capacity in brain:

Levels of brain MDA (CAT. No. MD2529) and TAC (CAT. No. TA2513) were estimated according to the procedures adopted by Ohkawa et al. (1979) and Koracevic et al. (2001), respectively; using colorimetric diagnostic kits purchased from

Biodiagnostic Company (Dokki, Giza, Egypt).

7. Histopathological Examination:

Following the method of Bancroft and Layton (2013), the formalin-fixed brains were washed under tap water for one hour, sliced, processed in different concentrations of alcohol solutions, and embedded into paraffin blocks. Using microtome, the paraffin blocks were cut into 3-µm sections, and then were stained with haematoxylin and eosin stain for microscopic examination. Microscopically, sections were semi-quantitatively scored as follows: (–): absent; (+): mild < 25%; (++): moderate < 50%; (+++): severe > 50% of examined sections (AbuBakr et al., 2018).

8. Immunohistochemical Investigation:

The immunohistochemical investigations of GFAP and HSP70 were conducted according to El-Bialy et al. (2017). The formalin-fixed brain sections were deparaffinized and then rehydrated in alcohol. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 15 min at room temperature. Antigen retrieval was done in citrate buffer (Ph 5.4) for 20 min. At 4 °C, primary antibody overnight incubation was done with GFAP (dilution 1: 500) and HSP70 (dilution 1: 300), which were obtained from Santa Cruz Biotechnology, California, USA. Sections were washed with phosphate saline, and then incubated with a secondary labelled polymer (Dako, California, USA) at room temperature for 30 min. Sections subjected to diaminobenzidine (DAB, Sigma Company, USA) for 2 min and then stained with haematoxylin.

Semi-quantitative analysis of GFAP and HSP70 was performed by counting the average values of 10 random high-power

fields (HPFs) as described by Tahoun *et al.* (2021). Immune labelling was (–): negative; (+): mild < 25%; (++): moderate < 50%; (+++): severe > 50% of all examined high-power fields.

9. Statistical analysis:

The obtained data were subjected to ANOVA followed by Duncan's Multiple Range test for post-hoc analysis using SPSS software, version 16 (released in 2007) and are presented as means ± SE. Statistical differences were set at *p* <0.05.

RESULTS

1. Effect of WBH on animals' behaviour and general health condition:

Rats exposed to a single bout of WBH showed increased respiratory rate, sweatiness, unsteadiness, and lethargy. The animals laid down without movement, even after gentle pushing, and this was followed by tremors and a deep coma. Among all the exposed rats, one animal exhibited colonic convulsions before death. Sweatiness, increased respiratory rate, unsteadiness, and lethargy were also observed in the subacute WBH group on the first day of exposure. The severity of these signs decreased with repeated daily exposure, except for one rat

that displayed severe daily manifestations and died on the 6th day of heat exposure.

2. Effect of WBH on animals' body temperature and body weight:

The effects of acute or subacute WBH on body temperature and body weight are illustrated in Table 1. A significant (*P<0.05*) increase in body temperature and a significant decrease in body weight were observed in both acute and subacute hyperthermic groups, compared to the normothermic group. Meanwhile, significant differences were recorded between acute and subacute hyperthermic groups. Rats in the AWBH group exhibited the highest body temperature, while rats in the SWBH group showed the lowest body weight values.

3. Effect of WBH on serum glucose and cortisol levels:

Compared to the normal control values, acute exposure of rats to single bout of hyperthermia for 4 hours induced significant (*P<0.05*) elevations in the serum glucose and cortisol levels, while exposure of rats to repeated bouts of hyperthermia, 2 hours for a week did not show any significant differences in the serum glucose and cortisol levels (Table 1).

Table 1: Heat-stress physiological indices in the normothermic and whole-body hyperthermic rats.

Parameters	NT	AWBH	SWBH
Body Temperature	36.25 ± 0.31[c]	41.38 ± 0.43 [a]	39.07 ± 0.25 [b]
Body Weight	159 ± 3.33 [a]	144 ± 1.65 [b]	119 ± 3.97 [c]
Glucose (mg/dL)	174 ± 4.04 [b]	200 ± 3.53 [a]	167 ± 6.34 [b]
Cortisol (µg/dL)	2.14 ± 0.588 [b]	5.96 ± 0.799 [a]	2.36 ± 0.504 [b]

Values are means ± SE (n = 5). Different letters (a, b, c) in the same column indicate significant differences at P < 0.05. NT: Normothermic, AWBH: Acute whole-body hyperthermic, SWBH: Subacute whole-body hyperthermic.

4. Effect of WBH on the oxidant/antioxidant biomarkers in the brain:

As displayed in Table 2, the rats exposed to single bout of WBH for 4 hours showed significant ($P<0.05$) increments in brain levels of 8-OHdG and MDA, concomitantly with a significant reduction in TAC, relative to the NT rats. However, rats exposed to repeated bouts of WBH for 2 hours for a week did not exhibit any significant changes when compared to the NT group.

Table 2: Brain oxidant/antioxidant biomarkers in the normothermic and whole-body hyperthermic rats.

Parameters	NT	AWBH	SWBH
8-OHdG (ng/g)	7.86 ± 0.493 [b]	14.88 ± 2.01 [a]	9.46 ± 0.619 [b]
MDA (nmol/ g)	12.76 ± 0.945 [b]	28.22 ± 1.14 [a]	15.90 ± 1.17 [b]
TAC (Mm/g)	37.05 ± 1.95 [a]	22.93 ± 1.51 [b]	34.27 ± 1.30 [a]

Values are means $\pm$ SE (n = 5). Different letters (a, b, c) in the same column indicate significant differences at P < 0.05. NT: Normothermic, AWBH: Acute whole-body hyperthermic, SWBH: Subacute whole-body hyperthermic, 8-OHdG: 8-hydroxy-2'-desoxyguanosine, MDA: Malondialdehyde, TAC: Total antioxidant capacity.

5. Effect of WBH on the brain histoarchitecture:

The histopathological changes in brains of the different groups are shown in Table 3 and Figure 1. Brain sections of the normothermic group showed normal cerebral (Fig. 1a) and cerebellar (Fig. 1d) histological structure. However, oedema, haemorrhage of leptomeninges, congestion of subarachnoid blood vessels, neuropil vacuolation, and degenerated neurons were observed in the cerebrum of both AWBH (Fig. 1b) and SWBH (Fig. 1c) groups. Meanwhile, various pathological alterations were recorded in brain cerebellum of AWBH (Fig. 1e) and SWBH (Fig. 1f) groups, including oedema, degeneration, and complete loss of Purkinje cells in Purkinje cell layer and neuropil vacuolation in molecular layer. Interestingly, the observed pathological changes were more pronounced in the AWBH group (Table 3).
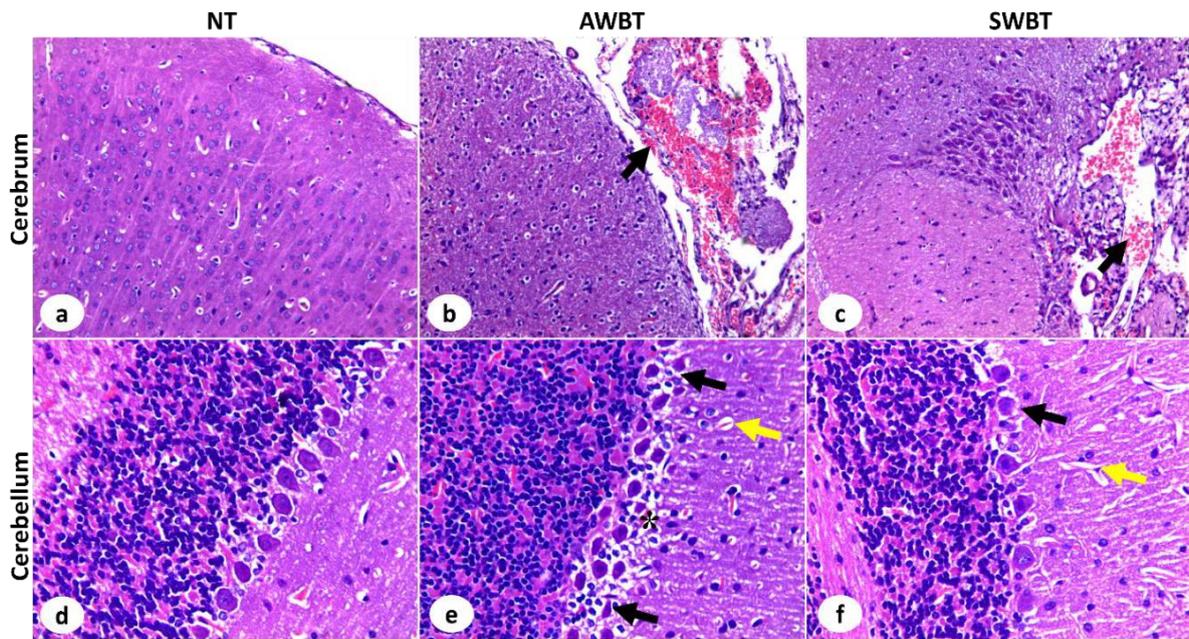
Fig. 1: Rat cerebrum; (a) Normothermic rat showing normal histology of meninges and cerebellar cortex. (b) Acute whole-body hyperthermic group showing meningeal haemorrhage (arrow), neuronal degeneration and oedema. (c) Subacute whole-body hyperthermic group showing meningeal blood vessels congestion (arrow), neuropil vacuolation and degenerated neurons. cerebellum; (d) Normothermic rat showing normal histology of brain cerebellar layers. (e) Acute whole-body hyperthermic group showing neuropil vacuolation in molecular layer (yellow arrow), degeneration and loss of Purkinje cells with oedema in Purkinje cell layer (black arrows). (f) Subacute whole-body hyperthermic group showing neuropil vacuolation in molecular layer (yellow arrow) and few degenerated neurons in Purkinje cell layer (black arrow). H&E, X10.

Table 3: The main histopathological lesions of the different treated groups.

Lesion	NT CB	NT CL ML	NT CL PcL	NT CL GcL	AWBH CB	AWBH CL ML	AWBH CL PcL	AWBH CL GcL	SWBH CB	SWBH CL ML	SWBH CL PcL	SWBH CL GcL
Haemorrhage	–	–	–	–	++	–	–	–	+	–	–	–
Congestion	–	–	–	–	+	–	–	–	+	–	–	–
Edema	–	–	–	–	+	–	+++	–	+	–	+	–
Neuropil vacuolation	–	–	–	–	+	+	–	–	+	+	–	–
Degenerated neurons	–	–	–	–	++	–	+++	–	+	–	+	–

The histopathological alterations are graded as follows: (–): absent; (+): mild; (++): moderate; (+++): severe. NT: Normothermic, AWBH: Acute whole-body hyperthermic, SWBH: Subacute whole-body hyperthermic, CB: cerebrum; CL: cerebellum; ML: molecular layer; PcL: Purkinje cell layer; GcL: Granular cell layer.

6. Effect of WBH on HSP70 and GFAP expressions in the brain:

Table 4 and Figure 2 show the immune reactivity for HSP70 in the brain tissues of normothermic and hyperthermic groups. Mild immunoreactivity for HSP70 was observed in the cerebrum (Fig. 2a) and cerebellum (Fig. 2d) of the NT group. On the other hand, the cerebrum of AWBH (Fig. 2b) and SWBH (Fig. 2c) groups exhibited severe and moderate immune reactivity for HSP70, respectively. While the AWBH group showed moderate immunoreactivity in the granular cell and Purkinje cell layers (Fig. 2e), mild immunoreactivity was observed only in the granular cell layer of the cerebellum of the SWBH group (Fig. 2f).
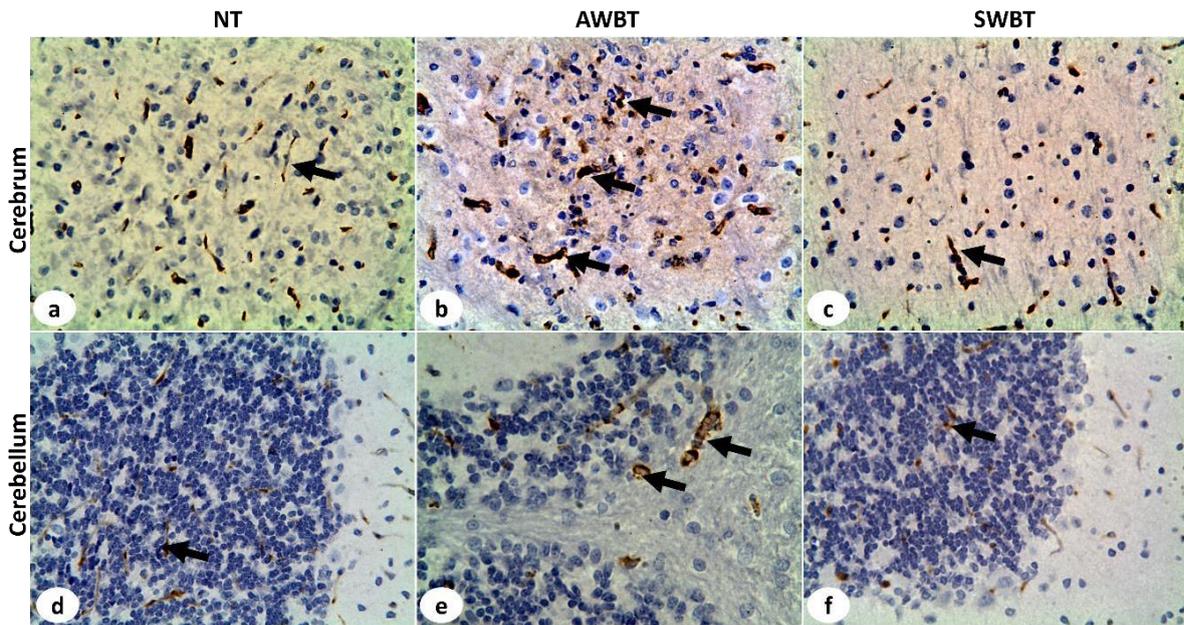

Fig. 2: HSP70 IHC (a-c); cerebrum showing mild, severe, and moderate immunolabelling (arrows) in normothermic, acute whole-body hyperthermic, and subacute whole-body hyperthermic groups, respectively. (d-f); cerebellum showing mild immunolabelling (arrow) in granular cell layer in normothermic (d) and subacute whole-body hyperthermic groups (f), and moderate immunolabelling in granular cell and Purkinje cell layers (arrows) in the acute whole-body hyperthermic group (e). Haematoxylin counterstained X20.

Regarding the immunoreactivity of GFAP displayed in Table 4 and Figure 3, mild immune reactivity was observed in the cerebrum (Fig. 3a) and granular cell layer of the cerebellum (Fig. 3d) of the normothermic group. Nevertheless, the cerebrum of the AWBH (Fig. 3b) and SWBH (Fig. 3c) groups exhibited severe and moderate immune reactivity for GFAP, respectively. Cerebellar astrocytes only in the granular layer of the cerebellum showed severe immune reactivity in the AWBH group (Fig. 3e), and mild immune reactivity in the SWBH group (Fig. 3f).
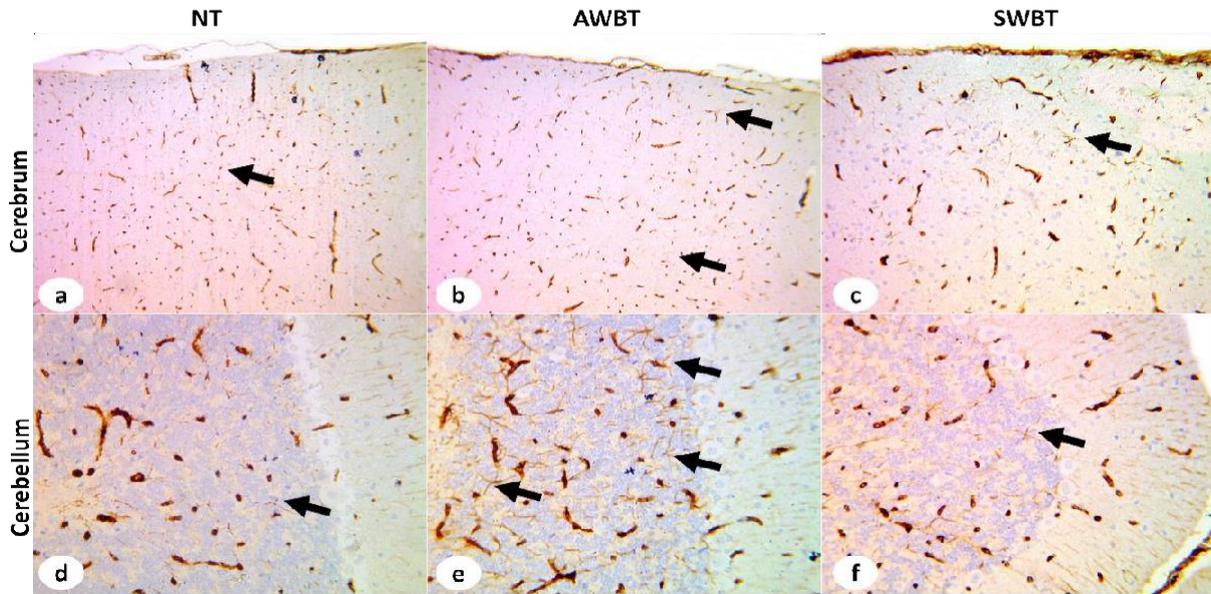
Fig. 3: GFAP IHC (a-c); Cerebrum astrocytes showing mild, severe, and moderate positive brown staining (arrows) in normothermic, acute whole-body hyperthermic, and subacute whole-body hyperthermic groups, respectively. (d-f); Cerebellum astrocytes showing mild, severe, and mild positive brown staining (arrows) in granular cell layer in normothermic, acute whole-body hyperthermic, and subacute whole-body hyperthermic groups, respectively. Haematoxylin counterstained X40.

Table 4: Semi-quantitative analysis of HSP70 and GFAP immuno-histochemical reactivity in the brains of different groups.

IHC	NT CB	NT CL ML	NT CL PcL	NT CL GcL	AWBH CB	AWBH CL ML	AWBH CL PcL	AWBH CL GcL	SWBH CB	SWBH CL ML	SWBH CL PcL	SWBH CL GcL
HSP70	+	−	−	+	+++	-	++	++	++	−	−	+
GFAP	+	−	−	+	+++	−	−	+++	++	−	−	+

The immune reactivity was graded as follows: (−): negative; (+): mild <25%; (++): moderate < 50%; (+++): severe > 50% of all examined high-power fields. NT: Normothermic, AWBH: Acute whole-body hyperthermic, SWBH: Subacute whole-body hyperthermic, CB: cerebrum; CL: cerebellum; ML: molecular layer; PcL: Purkinje cell layer; GcL: Granular cell layer, Hsp70: Heat shock protein 70, GFAP: Glial fibrillary acidic protein.

DISCUSSION

Hyperthermia and the associated heat stress-related disorders have recently increased as a result of global warming and the world-wide increase in the intensity, frequency, and duration of heat waves (Williams, 2020). Despite the therapeutic value of mild hyperthermia, a worse prognosis is associated with hyperthermia with a core body temperature of 38.5 °C (Lee *et al.,* 2012).

Exposure to HS has been reported to cause numerous physiological, behavioural, biochemical, and pathological disorders (Lee *et al.,* 2015; Brugaletta *et al.,* 2022). Accumulating evidence has suggested that the central nervous system is extremely sensitive to hyperthermia, especially with

excessive or prolonged exposure (Walter and Carraretto, 2016).

Elevated core body temperature, heart rate, and sweat production are common physiological responses to HS as a consequence of autonomic nervous system activity (Iguchi *et al.*, 2012). Indeed, further postmortem investigations are needed as forensic evidence for exposure to hyperthermia and to give a better diagnosis of heat-related deaths. Therefore, this investigation aimed to study the effects of exposure to single or repeated WBH, focusing on the substantial role of different postmortem investigations for identifying adaptation responses to hyperthermia and the diagnosis of heat-related deaths.

To evaluate the effect of acute long duration-versus subacute short duration-exposure to WBH, this study investigated well-known heat stress indices, including body temperature, body weight, and the serum glucose and cortisol levels. Acute or subacute exposure to WBH induced significant increases in body temperature and significant decreases in body weight, whereas the highest body temperature was observed in the AWBH-exposed rats and the lowest body weight was recorded in the SWBH-exposed rats. Moreover, the serum glucose and cortisol levels were significantly elevated only in the AWBH group.

Consistent with our findings, Lee *et al.* (2015) revealed that exposure of mice to a high temperature of 43 °C for 15 min, increased the core body temperature and decreased the body weight. Moreover, increases in rectal body temperatures and decreases in weight gains were recorded in mice following a daily 60 min exposure to HS between 34 °C and 38.5 °C for 2 weeks (Harikai *et al.*, 2003). Rats exposed daily to a

high temperature (41°C) for 2 weeks showed a significant reduction in feed intake after one week of exposure, while body weight significantly reduced after 2 weeks of exposure (Qari *et al.*, 2021).

The observed reduction in the body weight of hyperthermic groups is closely linked to the recorded elevated rectal body temperature that could be resulted from the reduction in feed intake as an adaptive physiological response to decrease the heat of digestion and the heat of metabolism (Magdub *et al.*, 1982). The reduction in feed intake depends primarily on the intensity and duration of heat exposure (Ominski *et al.*, 2002). Heat stress markedly affects the post absorptive protein, carbohydrate, and lipid metabolism (Baumgard and Rhoads, 2013). Also, Ma *et al.* (2021) found that exposure of broilers to HS for a week caused elevation of corticosterone level, resulting in reduction of the protein synthesis and increase of protein breakdown.

Heat stress also affects metabolic regulation of glucose as evidenced in this study by the significant elevation in serum glucose level in the AWBH group without any significant changes in the SWBH group. Similarly, acute bout of whole-body heat stress elevated serum glucose level (Kimball *et al.*, 2018) that may be attributed to the rapid depletion of hepatic glycogen content and the intensive glycogenolysis in response to HS (Miova *et al.*, 2014).

The hypothalamus-pituitary-adrenal (HPA) axis is the main neuroendocrine system in response to various stressors (Atkinson *et al.*, 2006). In response to stress, HPA axis is activated, resulting in elevation of the circulating adrenocorticosteroid hormone, and consequently the serum cortisol level, which is a curial indicator for

the degree of stress (Dong and Liu, 2013). Our findings revealed significant elevation in the serum cortisol level in the AWBH group. In consistence with our findings, previous literatures confirmed that acute heat stress is associated with increase in the serum cortisol levels in rats (Jasnic *et al.,* 2010; Wang *et al.,* 2015) and mice (Lee *et al.,* 2015) that could be attributed to the activation of the HPA axis in response to hyperthermia (Mete *et al.,* 2012).

Our findings demonstrated that exposure to WBH resulted in disturbance in the oxidant/antioxidant balance as indicated by a significant increment in MDA level and a significant reduction in TAC in brain tissue of rats exposed to single bout of WBH for 4 hours. Mounting evidence has reported that oxidative stress is involved in hyperthermia-induced multi-organ dysfunctions (Gupta *et al.,* 2018; Chauhan *et al.,* 2021; Qari *et al.,* 2021; Zhao *et al.,* 2021). Importantly, the recorded disturbance in the oxidant/antioxidant balance in the brain of hyperthermic rats may be attributed to the excessive production of free radicals that results in lipid peroxidation, and consequently overutilization of the intracellular antioxidants. The intensity of lipid peroxidation and the marked consequences of increased free radicals are directly linked to the extent of DNA fragmentation (Martins *et al.,* 2021). Herein, the oxidative brain injury induced by exposure to WBH extended to induce oxidative DNA damage that was reflected by the increase of 8-OHdG levels in the brain of the hyperthermic rats. In the same line, heat shock-driven oxidative stress induced DNA damage and increased 8-OHdG level in cells/tissues in oysters (Rahman *et al.,* 2022). Moreover, increased serum 8-OHdG level

was observed in high temperature-exposed bakery workers (Kheder and Al-Dosky, 2023).

In association with the obtained biochemical findings, whole-body hyperthermia-exposed rats exhibited several pathological changes, including oedema, congestion, haemorrhage, and neuronal degeneration in cerebrum, along with severe oedema around Purkinje cells in cerebellum as previously reported by Sharma and Hoopes (2003); Rebez *et al.* (2023), suggesting that brain oedema is the most prominent finding associated with hyperthermia. Exposure to HS causes damage to the blood–brain barrier with gradual leakage of ions and proteins into brain tissue, promoting brain oedema (Sharma, 2006). Indeed, the upregulation of the constitutive and inducible nitric oxide synthase in the brain of rats exposed to acute HS at 38 °C for 4 h may be the main cause of blood–brain barrier damage, brain oedema, and cell injury (Sharma *et al.,* 2000; Sharma, 2006). Importantly, brain oedema not only damages brain function via increasing intracranial pressure and brain volume, tissue softening, and vital center compression, but it also causes secondary brain cell and tissue injury (Sharma, 2006; Sorby-Adams *et al.,* 2017).

Our findings revealed haemorrhages only in the AWBH-exposed rats, which is compatible with (Sharma and Hoopes, 2003), who reviewed severe and diffuse leptomeningeal haemorrhages in short-duration heat stress. The cerebellar pathological changes were mainly observed in the Purkinje cell layer, with little or no change in the granular and molecular layers, as recorded by Sharma and Hoopes (2003), indicating the selective vulnerability of

cerebellar Purkinje cells to thermal injury (Bazille *et al.,* 2005). Not surprisingly, all the observed pathological changes were more pronounced in the AWBH-exposed rats, reflecting that acute exposure to a single bout of HS is more dangerous than repeated exposure.

Among the heat stress markers, HSP70 is the ideal cellular marker for examining the heat response of the hyperthermic groups. Immunohistochemically, HSP70 is markedly expressed, in various degrees, in the brain cerebrum and cerebellum of the hyperthermic groups. This finding agrees with that recorded by Kim *et al.* (2013), who found elevation in HSP70 expression in the brain of mice exposed to HS at 43 °C, 15 min for 14 days and decreased to normal level after 21 days.

Heat shock proteins (HSPs) are normally located in the cells in low or extremely low concentrations, even without exposure to certain stresses. In normal physiological states, HSPs bind together with the heat shock factor (HSF), a transcriptional regulator of HSPs (Doberentz *et al.,* 2017). Exposure to numerous stress factors, particularly thermal stress causes protein degradation and damage (Horowitz and Robinson, 2007). In such case, HSPs rapidly separate from the HSF to bind with the degraded protein, resulting in upregulation of new HSPs for maintaining the cellular protein homeostasis and prohibiting apoptotic cell death (Kim *et al.,* 2020).

The overexpression of HSP70, a member of HSPs, is considered a brain marker of thermal stress. Although the upregulation of HSP70 induced by HS has a cytoprotective effect, prolonged

hyperthermia or temperatures exceeding 42°C have direct cytotoxic effects and cause protein denaturation (Doberentz *et al.,* 2017). Accordingly, the marked immunostaining of HSP70 in the brain tissue of the hyperthermic groups may be attributed to the cellular damage caused by hyperthermia and the increase in its expression, which directly correlates with the intensity and duration of HS.

Additionally, various degrees of GFAP expression were observed in cerebrum and cerebellum of hyperthermic groups. Similarly, immunohistochemical examination revealed a time-dependent increase in the GFAP-stained astrocytes in the hippocampus of mice exposed to HS at 43 °C, 15 min for 7, 14, or 42 days (Lee *et al.,* 2015). Furthermore, Sharma *et al.,* (1992) recorded marked increase in the GFAP immunoreactivity in particular regions of the brain of rats exposed to HS at 38 °C for 4 h. Conversely, the same literature reported no GFAP immunoreactivity at the same temperature for 1 h or 2 h, or at a lower temperature of 36 °C for 4 h.

Astrocytes are sub-type of glial cells that have a substantial role in maintaining CNS homeostasis and regulating its functions (Kumar *et al.,* 2023). Reactive astrogliosis is the response of astrocytes to numerous mechanical, physical, or chemical brain disorders that could be indicated by the over expression of the GFAP in brain (Moulson *et al.,* 2021). GFAP, a cytoplasmic soluble protein in the astrocytes, is a pivotal indicator for astrocytes proliferation in response to various brain injuries (Cikriklar *et al.,* 2016). Heat stress is one of the most common causes of glial cells activation that release proinflammatory cytokines and neurotoxic factors, thereby, causing neuronal

death (Block *et al.,* 2007; Lee *et al.,* 2015). Herein, the increase in the immunoreactivity of GFAP in the cerebrum or cerebellum of hyperthermic rats suggests glial cells activation and neuronal damages that depends on the intensity of the tissue damage.

Taken together, the above-mentioned biochemical, pathological, and immunohistochemical findings correlate with the core body temperatures of rats measured immediately before animals sacrificing. Notably, marked changes were observed in rats exposed to a single bout of WBH for 4 h than those exposed to repeated bouts of WBH 2 h daily for 7 successive days that may be explained by the physiological adaptation on repeated HS exposures (Williams, 2020). Importantly, Tyler *et al.* (2016) reported that physiological adaptations are noticeable after a week or two of HS exposure and may have a significant effect on the response to HS. New evidence suggested that short-term heat acclimation for about 7 days is valuable for people works under HS circumstances (Foster *et al.,* 2020). This suggests that the duration of heat exposure is an essential determining factor for the consequences of hyperthermia-induced brain injuries.

CONCLUSION

Our data revealed that exposure to acute hyperthermia for a single bout of 4 hours markedly increased core body temperature, decreased body weight, elevated blood glucose and stress hormone levels, altered oxidant/antioxidant status and the histological structure of the brain, and up-regulated HSP70 and GFAP expressions more than the repeated daily exposure to high temperature for 2 hours over 7 successive days, providing evidence of the

thermotolerance of rats to the repeated subacute exposure to WBH. Importantly, this study may present a new strategy in forensic medicine settings for the reliable antemortem and postmortem diagnosis of heat-related deaths.

STATEMENTS & DECLARATIONS
Ethics approval and consent to participate: Ethics approval and consent to participate this study was approved by the International Animal Care and Use Committee IACUC, Faculty of Veterinary Medicine, University of Sadat City (Approval No. VUSC-019-1-21).
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